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Determination and quantification of red blood cell populations in samples

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TITLE: DETERMINATION AND QUANTIFICATION OF RED BLOOD CELL POPULATIONS IN SAMPLES

The invention relates to the detection and determination of erythrocytopathies and hemoglobinopathies.

The detection of circulating fetal cells in maternal blood samples represents an important area of laboratory support to the obstetrical management of women. Although the concentration of fetal erythrocytes found in the maternal blood circulation during pregnancy is mostly very small and without a clear clinical significance in many cases, substantial hemorrhage may result from a number of causes including fetal or maternal trauma and placental defects (1). The quantification of fetal red blood cells (RBC's) is most commonly used to estimate the degree of feto-maternal hemorrhage (FMH). Either in cases of trauma with suspected placental injury or in the situation of a RhD incompatibility between the fetus and the mother for prevention of hemolytic disease of the new-born (HDN) during pregnancy (2, 3). The obstetrical management of women includes the prevention of mother immunization against a foreign fetal cell antigen, and the monitoring of maternal antibody concentration. To prevent an immune response, an immunoprophylaxis based on anti-RhD polyclonal antibodies, is given to the mother at a dose proportional to the estimated count of fetal RBC's present in the maternal blood circulation (4, 5). It is therefore important to be able to at least semi-quantify the relative amount of said cells.

Most clinical laboratories perform FMH estimates on the basis of variations of the slide-based microscopic counting method of acid elution originally described as the Kleihauer-Betke test (6). Although this assay has proven to be clinically useful in the detection of large episodes of FMH requiring maternal treatment with more than the standard dose of Rh immune globulin, it is laborious and suffers from subjectivity and imprecision (7, 8). Besides the experience of the laboratory technicians for interpretation of the results, the test has a tendency to overestimate the size of feto-maternal hemorrhages because maternal HbF containing RBC's or F-cells are counted within the population of fetal cells (9).

Several alternative and more accurate screening methods to detect FMH using flow cytometry have been proposed and described. The first reports investigating the

feasibility of using flow cytometry for fetal cell counting primarily relied upon the detection of the human D antigen on the cell surface of RBC's (10, 11, 12, 13). These approaches all demonstrated greater sensitivity and precision than manual methods. However, the use of anti-RhD is applicable only to the clinical situations with Rh or D antigen incompatibility and can not be utilized in all cases of maternal trauma and suspected FMH. Several other methods for flow cytometric detection of fetal cells in maternal peripheral blood have recently been described. The methods differ in their means of using various cellular fixation and permeabilization steps, usually in combination with the intracellular detection of fetal hemoglobin (HbF) antigen using anti-HbF antibodies.

As an increase of the expression of fetal hemoglobin (HbF) in peripheral red blood cells is also a common feature in hemoglobinopathies comprising genetic disorders of hemoglobin such as sickle-cell disease and β-thalassemia. (14, 15, 16, 17), a method for the detection of HbF in blood cells also finds its use in the diagnosis of hemoglobinopathies other than those related to FHM .

The invention provides a method for distinguishing between subsets of red blood cells in a sample comprising contacting said sample with at least a first marker reagent reactive with a first, preferably, antigenic component of a red blood cell and with at least a second marker reagent reactive with a second, preferably antigenic, component of a red blood cell and determining the reactivity of said markers with said cells. In a preferred embodiment, the invention provides a method for distinguishing between and/or quantification of various subsets of erythrocytes in a sample comprising the use of at least two markers reactive with at least two subsets of red blood cells, such as HbF containing erythrocytes (F-cells) or fetal versus adult red blood cells in blood. To diagnose or assess FMH, the invention thus provides for distinguishing between subsets of red blood cells in a sample comprising combining testing for a determinant of essentially fetal cells with a determinant for essentially adult cells. Of course, the various subsets may overlap in that some cells in each subset carry two of the markers used in the method chosen. Said method is most useful to distinguish between subsets of mature erythrocytes, i.e. those that have matured beyond the nucleated RBC or immature reticulocyte phase. In one embodiment, the invention provides a method for distinguishing between and/or quantification of fetal red blood cells (RBC's) in maternal

blood comprising the use of at least two markers reactive with various subsets of red blood cells. In another embodiment, the invention provides a method for distinguishing between and/or quantification of adult HbF containing RBC's in blood comprising the use of at least two markers reactive with various subsets of red blood cells. In spite of the recent reported results of the detection of fetal HbF containing cells in different maternal blood samples, the use of a single parameter does not offer an accurate and reliable quantification of fetal RBCs and maternal F cells. A dual or multiple marker approach has several advantages. Although the use of the HbF antigen as single marker allows broad application for fetal red blood cell detection to many clinical situations, the use of anti-HbF by itself provides the possibility of an overestimation of the proportion of true fetal cells in a given HbF population.

Use of the intracellular cell marker Carbonic anhydrase (CA) for adult RBC's (18) in combination with HbF should allows for example the clear distinction of fetal red blood cells, from possible interfering maternal F cells that have a lower cellular HbF content and are positive for the CA marker. Small populations of adult erythrocytes containing HbF are found in individuals of any age; these cells have been termed F cells. For people with sickle cell anemia, these cells are functionally quit important because they are capable of transporting and releasing oxygen.

A method for distinguishing between subsets of red blood cells in a sample comprising contacting said sample with at least a first marker reagent reactive with a first component of a red blood cell and with at least a second marker reagent reactive with a second component of a red blood cell and determining the reactivity of said marker reagents with said cells. Suitable cell surface components to be detected are for example: CD71, a type II membrane glycoprotein of 90-95 kDa that exists as a homodimer on most dividing cells including RBCs. The protein plays a critical role in the uptake of iron, through the binding and endocytosis of transferrin, the major iron-carrying protein.; GpA, a cell surface sialoglycoprotein of 41 kDa is exclusively expressed on human erythroid cells and their progenitors. The protein is clinically important in the classification of acute leukemias.; i, a glycosilated Lewis antigen structure expressed on adult T- and B-lymphocytes and fetal lymphocytes and RBCs during the first 8 months of development. For some applications, such as flow cytometric detection it is preferred that at least one of the components to be detected comprises an intracellular component. Suitable intracellular components are for example: HbE, a intracellular hemoglobine protein consisting of 4 protein subunits of approx. 140 a.a. The embryonic hemoglobine

tetramer futher consists of different polypeptide chains, ε and ξ or ε and α. The expression of HbE is most prominent in embryonic red blood cells; CA, Carbonic anhydrases (carbonate dehydratase; carbonate hydrolyase) form a large family of genes encoding zinc metalloenzymes of great physiologic importance. As catalysts of the reversible hydration of carbon dioxide, these enzymes participate in a variety of biologic processes, including respiration, calcification, acid-base balance, bone resorption, and the formation of aqueous humor, cerebrospinal fluid, saliva, and gastric acid. CAs are encoded by members of 3 independent CA gene families, i.e., alpha-CA, beta-CA, and gamma-CA. Genes in the alpha-carbonic anhydrase family encode either active carbonic anhydrase isozymes or 'acatalytic' (i.e., devoid of CO2 hydration activity) carbonic anhydrase-related proteins. Alpha-carbonic anhydrases show extensive diversity in tissue distribution and in their putative or established biologic functions. Some of the alpha-CAs are expressed in almost all tissues (e.g., CA 2), whereas some show a more restricted expression, such as CA 1 in erythrocytes. In cells, they may reside in cytoplasm, in mitochondria, or in secretory granules, or associate with membranes. Erythrocyte carbonic anhydrase has 2 isoenzymes with different amino acid sequences and specific activities. B and C were the original designations for these 2 major forms which later were called CA I (or A) and CA II (or B), respectively. In cells, they may reside in cytoplasm, in mitochondria, or in secretory granules, or associate with membranes.

Such an intracellular component to be detected can be the whole component per se, or can for example be the intra-cytosolic part of a protein or receptor that otherwise projects or extends through the cell-membrane. For intracellular detection of intracellular components, fixation and permeabilization of the red blood cells is required, this advantageously provides rigidity and stability to the erythrocytes to be identified; detecting intracellular antigens in fixed erythrocytes thus allows for less background noise than detecting extracellular antigens in non-fixed cells only. A preferred method is using a combination of two intracellular components (target proteins or antigens) to discriminate between different red blood cell populations of for example fetal, parental or adult origin.

In a preferred embodiment, the invention provides a method wherein said first component consists of hemoglobin F. Hemoglobine (Hb) is a intracellular protein consisting of 4 protein subunits of approx. 140 a.a. The fetal hemoglobine tetramer futher consists of different polypeptide chains, γ and ξ or γ and α. The expression of HbF

is essentially fetal, in that it is most prominent in fetal red blood cells, but is also in low concentrations present in adult red blood cells. HbF is red blood cell specific. Because of the precise discrimination between the cell populations with HbF and another intracellular protein, the invention provides a method for nearly truly quantifying the true fetal cells and not to include or to count possible interfering adult F cells. In another preferred embodiment, the invention provides a method wherein said second component consists of carbonic anhydrase B. Carbonic anhydrase is protein or metalloenzyme with a catalytic activity for CO_2 The expression of CA is essentially or pre-dominantly in adult cells. Much preferred (and further explained in the detailed description herein) is a method wherein said first component consists of hemoglobin F and said second component consists of carbonic anhydrase , especially wherein said carbonic anhydrase is of type I.

A marker reagent used in a method according can comprise any kind of binding molecule, such as phage derived binding molecules (sometimes also called phage antibodies), but preferred is a method wherein at least one of said marker reagents comprises an antibody. An antibody, in the format of complete Ab or Fab, Fv, sFv, camel derived single chain or other protein structure, is a protein that comprises a so-called light chain and/or a heavy chain, chains, which are each or in combination are responsible for the specific binding of the target antigen. A particularly useful anti-HbF antibody is specific for the γ protein subunit of hemoglobin F. Antibodies raised against the i antigen are most often specific for this surface protein in the glycosilated form. Monoclonal antibody CD71 is directed towards the transferrin receptor, while anti-GpA antibodies recognize a epitope on the Glycophorin A antigen. Another useful antibody is specific for the ε polypeptide chain of embryonic hemoglobin (HbE). Antibodies raised against CA isotypes are specific for the several epitopes present on the different carbonic anhydrases. It is of course preferred for ease of detection that least two or all of said marker reagents comprise an antibody, each of said antibodies being reactive with a distinct antigenic component of a red blood cell. After fixation and permeabilization the antibody will enter the cell and bind to the intracellular fixed target protein

Marker reagents, being antibodies, or other binding molecules, are most easily detected when labeled. Useful is detection via a label that comprises a fluorochrome. A fluorochrome to be used can be any of the known molecules used in flow cytometry and or microscopy. Examples of fluorochromes are; protein labels like R-PE, APC, GFP, and chemical labels like, Alexa dyes, Cy dyes, tandem labels between the mentioned

dyes, or others. Most of the dyes can be purchased from Molecular Probes or similar companies. . Preferred is a method wherein at least two of said marker reagents comprise a fluorochrome, each of said fluorochromes having a distinct emission spectrum. Emission spectra preferably range from 350 to 800 nm. Enzymes (peroxidase, alkaline phosphatase and others) are generally naive or recombinant proteins which can be also attached to antibodies or other marker reagents (labeled) and used to visualize color development with different fluorescent or non-fluorescent substrates like, ELF, TMB and others which can be purchased from Molecular Probes, Pierce and others.

Preferred is a method as provided herein further comprising determining the reactivity of said marker reagents with said cells by flow cytometry, i.e. by detecting said reactivity of said marker reagents with said cells by detecting fluorescence.

The invention also provides a diagnostic kit suitable for the differentiation of subsets of erythrocytes, said kit at least comprising a first marker reagent reactive with a first component of a red blood cell and a second marker reagent reactive with a second component of a red blood cell, preferably wherein said first component consists of hemoglobin F and/or wherein said second component consists of carbonic anhydrase. Type I carbonic anhydrase is preferred for reasons explained above. Said marker reagent is preferably an antibody, but can be in essence any binding molecule with the desired binding specificity, said antibody or binding molecule being reactive with a distinct, preferably intracellular and antigenic component of a red blood cell. Said kit can also comprise the desired fluorochrome or several distinct fluorochromes having a distinct emission spectrum.

The invention also provides a reagent mixture suitable for inclusion in such a kit and suitable for the differentiation of subsets of erythrocytes, said reagent mixture at least comprising a first marker reagent reactive with a first component of a red blood cell and a second marker reagent reactive with a second component of a red blood cell, preferably wherein said first component consists of hemoglobin F and/or wherein said second component consists of carbonic anhydrase. Type I carbonic anhydrase is preferred for reasons explained above.

In the detailed description, we report on a highly accurate and quantitative procedure for the detection of distinct populations of fetal and F cells by flow cytometry that can be routinely performed. The description further illustrates that the use of a dual fluorescent parameter assay, based on the combined detection of fetal hemoglobin (HbF)

and carbonic anhydrase (CA), is able to separate true fetal RBCs from interfering maternal F cells.

Furthermore, as an increase of the expression of fetal hemoglobin (HbF) in peripheral red blood cells is also a common feature in hemoglobinopathies comprising genetic disorders of hemoglobin such as sickle-cell disease and β-thalassemia, the invention also provides a method for the detection of HbF in red blood cells in samples of a subject with such a hemoglobinopathy.

Figures

Figure 1

The cytograms of figure 1 demonstrate the population of contaminating autofluorescent WBCs which can be detected and separated by the DNA staining dye LDS 751.

Figure 2

The cytograms of figure 2 are representative of a 10 % of cord blood mixed with normal adult blood and stained with monoclonal antibody directed to HbF. The distributions of fetal HbF+++ cells, adult HbF+ cells and adult HbF- cells are indicated in the different cytograms. The interfering population of adult F cells (HbF+) are difficult to separate from the fetal RBCs in R10 and negative stained adult cells (HbF-) in 4, as demonstrated in Figure 2D.

Figure 3

The cytograms of figure 3 are representative of a 5 % of cord blood mixed with normal adult blood and stained with antibodies directed to HbF and erythrocyte specific CA1. The distributions of fetal HbF+++ cells, fetal HbF++/CA++ cells, adult HbF+/CA++ or F cells, and adult HbF--/CA++ cells, are indicated in the different quadrants of the final cytogram. Figure 3D demonstrates the separation of the infering adult F cells (R8) from the true fetal cells in R10.

Detailed description

Several alternative and more accurate screening methods to detect FMH using flow cytometry have been proposed and described. The first reports investigating the feasibility of using flow cytometry for fetal cell counting primarily relied upon the detection of the human D antigen on the cell surface of RBC's (10, 11, 12, 13). These approaches all demonstrated greater sensitivity and precision than manual methods. However, the use of anti-RhD is applicable only to the clinical situations with Rh or D antigen incompatibility and can not be utilized in all cases of maternal trauma and suspected FMH. Several other methods for flow cytometric detection of fetal cells in maternal peripheral blood have recently been described (14, 15, 16, 17). The methods differ in their means of using various cellular fixation and permeabilization steps, in combination with the intracellular detection of HbF antigen using anti-HbF antibodies. The flow cytometric anti-HbF approach has several potential advantages and the HbF antigen allows broad application of flow cytometric fetal red blood cell detection to many clinical situations. Furthermore, the anti-HbF method provides good correlation with the standard Kleihauer-Betke test of fetal cell detection, although with a much higher precision than the manual acid elution assay. However, our findings with the approach of one cell marker such as HbF indicated that this single color assay was not precise enough for the enumeration of true fetal cells and could not rule out the inclusion of a low percentage of false-positive F cells. A small population of 2 to 8% adult cells containing a low amounts of HbF. Separation and a clear distiction of both populations of fetal HbF containing cells and interfering adult F cells with a lower HbF content is very important to produce accurate data of true fetal cell frequency in maternal blood for FMH assessment or F cell measurements.

Our findings describe an alternative approach to flow cytometric quantification of fetal RBC's using antibodies to the intracellular located Fetal Hemoglobin (HbF) and Carbonic anhydrase (CA), and an optimal intracellular staining technique. The method or procedure is based on the dicrimination between fetal and adult red blood cells using specific anti-Carbonic anhydrase (CA) polyclonal and monoclonal anti-HbF antibodies. The HbF antigen/protein is the best marker for the detection of fetal red blood cells, while the CA antigen/protein is a good marker for adult red blood cell and is almost absent in fetal red blood cells. The monoclonal and polyclonal antibodies used in the test are fluorochrome-conjugated for direct and indirect detection of HbF and CA markers in

a dual flow cytometry analysis. Both antibody preparations were shown to be specific for HbF or CA using the protocol as described in the material section. No positive cell staining was ever demonstrated without permeabilization of the cells.

The preliminary results from the dual-color flow cytometric method were comparable to those in some previous reports describing anti-HbFdata. The F cell percentages in whole blood of normal donors obtained during the study were also comparable to those in previous reports. Our findings demonstrate that, by adding a second unique cell marker such as Carbonic anhydrase to the HbF flow cytometric method, it is possible to identify distinct populations of red blood cells. The new method is able to discriminate between true fetal cell and possible false-positive F cells, by shifting the small population of HbF containing F cells from the fetal cell population to the larger population of CA positive adult cells. The complete dual-color staining and analysis of up to 5 samples can be easily performed within 1.5 hour of blood collection. The accurate detection of HbF containing fetal red blood cells in maternal blood circulation will aid to estimate the degree of feto-maternal hemorrhage (FMH) in women during pregnancy, and subsequently in the management of hemolytic disease of the new-born (HDN).

Finally, the new and sensitive flow cytometric method using a dual fluorescent staining procedure, will accurately identify and quantify both interfering maternal F cells and fetal red blood cell populations. In agreement with other studies (14, 15, 16, 17), it represents a practical and technically superior alternative for the routine measurement feto-maternal hemorrhage compared to the more subjective and manual Kleihauer-Betke test.

Materials and Methods

Specimen Collection

Whole blood of 0.5 to 1 ml from normal donors as well as cord blood were collected on EDTA. The samples were usually assayed on the same day or stored at 4 – 8 °C for 1 week before testing.

Antibodies and Reagents

Monoclonal antibody NaM16-2F4(mouse IgG1) specific for the γ chain of human HbF was previously described (14) and purchased from Bioatlantic (Nantes, France). Pure antibody preparations were directly conjugated to R-phycoerythrin (R-PE) following standard labeling procedures. Its PE-conjugated IgG1 isotype control was purified and

labeled using standard labeling procedures. Polyclonal goat anti-human carbonic anhydrase was purchased from AbCAM while the FITC-conjugated anti-goat was obtained from Sigma. LDS 751, DNA staining dye was purchased from Molecular Probes and stored at 4^{0}C until use. Newborn calf serum was purchased from Greiner All other reagents were of analytical grade.

Cell Fixation and Permeabilization

Ten microliters of whole blood or cord blood were resuspended in to 100 μl of Newborn calf serum (NCS) in PBS, after which the RBC's were fixed with 100 μl of 20% paraformaldehyde in PBS, vortexed for 5 s, and incubated at room temperature (RT) for 30 min. After fixation the cells were washed once with 2 ml of PBS with heparin and resuspended in 100 μl of PBS with heparin. For permeabilization of the RBC's, 100 μl the fixed cells were mixed thoroughly with 100 μl of 0.3% sodium dodecyl sulfate (SDS) in PBS with heparin and allowed to stand at RT for 3 min. To remove the SDS, the cells were washed twice with 2ml of PBS with heparin and suspended in 1 ml of the same solution.

HbF and CA Detection by Flow cytometry

For immunophenotyping, 100 μl of the washed cell suspension was then mixed with either 50 μl anti-HbF-PE MoAb diluted at 40 ug/ml in PBS, 50 μl of anti-Carbonic Anhydrase PoAb diluted 1-in-500, and 50 μl of LDS 751; or 100 μl of the washed cell suspension was mixed with 50 μl of LDS 751 and 50 μl of PBS and 50 μl isotype control as a negative control. After an incubation of 15 min. at RT in the dark, cells were washed once with 2 ml PBS containing heparin. The in 100 μl suspended cell solutions were both mixed with 50 ul of anti-Goat IgG-FITC and incubated at RT for 15 min. The cells were washed to remove the secondairy labeled conjugate and finally resuspended in 0.5 ml of PBS with heparin, ready for flow cytometry. The isotype control was used instead of the anti-HbF MoAb for a negative control.

Sample acquisition was performed on a Coulter Epics XL MCL flow cytometer (Beckman-Coulter, U.S.A.) The HbF and CA cells were counted by setting the autostop at 50.000 or 100.000 events, with the collection of measures of logFSC and logSSC, and fluorescence signals of logFL1, logFL2 and logFL4 as list mode files. Data analysis was performed with software (Winlist, Verity Software, Topsham, ME) on list mode files. The live gate on LDS 751 negative cells was used to exclude possible interfering nuclei-

containing white blood cells. The positive cutoff point was approximately at 0.5% above negative population of isotype control staining cells.

Results

A dual-color flow cytometric method results in the simultaneous detection of two intracellular antigens and provides a convenient and rapid test that can be completed within 1.5 hour of blood collection. The use of paraformaldehyde as fixative reagent and sodium dodecyl sulfate (SDS) to permeabilize fixed RBC's resulted in low background staining, a negligible HbF leakage, and minimal cell clumping. Following fixation and permeabilization both cell antigen markers could be detected with high fluorescence signals, which resulted in a clear distinction between different stained cell populations. An important aspect of the flow cytometric fetal cell count data is to exclude possible interfering adult hemoglobin containing RBC's and autofluorescent WBC's present from the region of fetal cell identification. The induced autofluorescent WBC's can be readily excluded from the fetal cell population by staining these nuclei-containing cells with a DNA staining dye such as LDS 751 as shown in Figure 1.

The unprecise separation of HbF containing F cells from the true HbF containing fetal cells is demonstrated in the cytograms of Figure 2. The fact that it is difficult to set a accurate region on the fetal cells without the interfering F cells, leads to the overestimation of the true population of fetal HbF containing cells. Especially when the number of fetal cells is low (0.4 – 0.6 %), the true count of fetal cells in a background of contaminating F cells with variable amounts of HbF is not accurate.

The flow cytometric results of the combination of the two different red blood cell markers HbF and CA, as presented in Figure 3, demonstrated that most fetal RBC's with a high HbF content and no CA could be well separated from interfering adult F cells with a lower HbF content but containing high amounts of CA. Adult RBC's with no HbF were as expected only positive for CA. Another small but not unexpected population of fetal cells, with a high HbF content and lower CA, was detected next to the fetal cell population and also clearly separated from the F cells. Both the populations of fetal cells combined together resulted in the true count of fetal RBC's in cord blood and mixtures of cord and adult blood.

The linearity and precision of the dual color flow cytometric method was studied in mixtures of cord blood and whole blood from non-pregnant adults. As shown in Figure 4,

excellent linearity for the method was observed by serial dilutions of mixtures of cord blood and adult blood (n=10), with a fetal cell frequency range of 0 to 5 %. Precision of the method was determined by performing an analysis on the same preparation of mixtures of cord and adult blood within a 5 day period. The precision of all the samples consistently resulted in a CV of < 5 %. Therefore, the new flow cytometric method demonstrated a excellent assay performance and was observed to be linear and precise both above and below the clinically important fetal cell frequency of approximately 0.6 %. Linearity of measurement was determined for samples consisting of different ratio's of cord blood mixed with normal adult blood in the range of 0.0 – 10 % positive cord blood cells (HbF/CA). Nine month old cord blood consists of 85 % fetal RBC's. The linear regression method was used to plot the know expected values versus the observed values for the percent of double labeled fetal cells determined by the double fluorescent cytometric method and resulted in a linearity of $y = 0.005x + 0.002$ whereby $R^2 = 0.9933$. Our studies indicate that the use of a second antibody for the detection of CA, preferably in combination with the HbF marker or with another determinant of essentially fetal cells, provides a more detailed discrimination of the different fetal and adult RBC populations and results in an improvement in the ability to determine fetal RBC frequency

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Claims

1. A method for distinguishing between subsets of red blood cells in a sample comprising contacting said sample with at least a first marker reagent reactive with a first component of a red blood cell and with at least a second marker reagent reactive with a second component of a red blood cell and determining the reactivity of said marker reagents with said cells.

2. A method according to claim 1 wherein at least one of said components comprises an intracellular component.

3. A method according to claim 2 wherein said first component consists of hemoglobin F.

4. A method according to claim 2 wherein said second component consists of carbonic anhydrase.

5. A method according to claim 2 wherein said first component consists of hemoglobin F and said second component consists of carbonic anhydrase.

6. A method according to anyone of claims 1 to 5 wherein at least one of said marker reagents comprises an antibody.

7. A method according to claim 6 wherein at least two of said marker reagents comprise an antibody, each of said antibodies being reactive with a distinct antigenic component of a red blood cell.

8. A method according to anyone of claims 1 to 7 wherein at least one of said reagents comprises a fluorochrome.

9. A method according to claim 8 wherein at least two of said marker reagents comprise a fluorochrome, each of said fluorochromes having a distinct emission spectrum.

10. A method according to anyone of claims 1 to 9 further comprising determining the reactivity of said marker reagents with said cells by flow cytometry.

11. A method according to anyone of claims 1 to 10 further comprising determining the reactivity of said marker reagents with said cells by detecting fluorescence.

12. A diagnostic kit suitable for the differentiation of subsets of erythrocytes, said kit at least comprising a first marker reagent reactive with a first component of a red blood cell and a second marker reagent reactive with a second component of a red blood cell.

13. A kit according to claim 12 wherein said first component consists of hemoglobin F.

14. A kit according to claim 12 wherein said second component consists of carbonic anhydrase B.

15. A kit according to claim 12 wherein said first component consists of hemoglobin F and said second component consists of carbonic anhydrase B.

16. A reagent mixture suitable for use in the differentiation of subsets of erythrocytes, said mixture at least comprising a first marker reagent reactive with a first component of a red blood cell and a second marker reagent reactive with a second component of a red blood cell.

Abstract

The invention relates to the detection and determination of erythrocytopathies and hemoglobinopathies. The invention provides a method for distinguishing between subsets of red blood cells in a sample comprising contacting said sample with at least a first marker reagent reactive with a first component of a red blood cell and with at least a second marker reagent reactive with a second component of a red blood cell and determining the reactivity of said marker reagents with said cells.

FIGURE 1

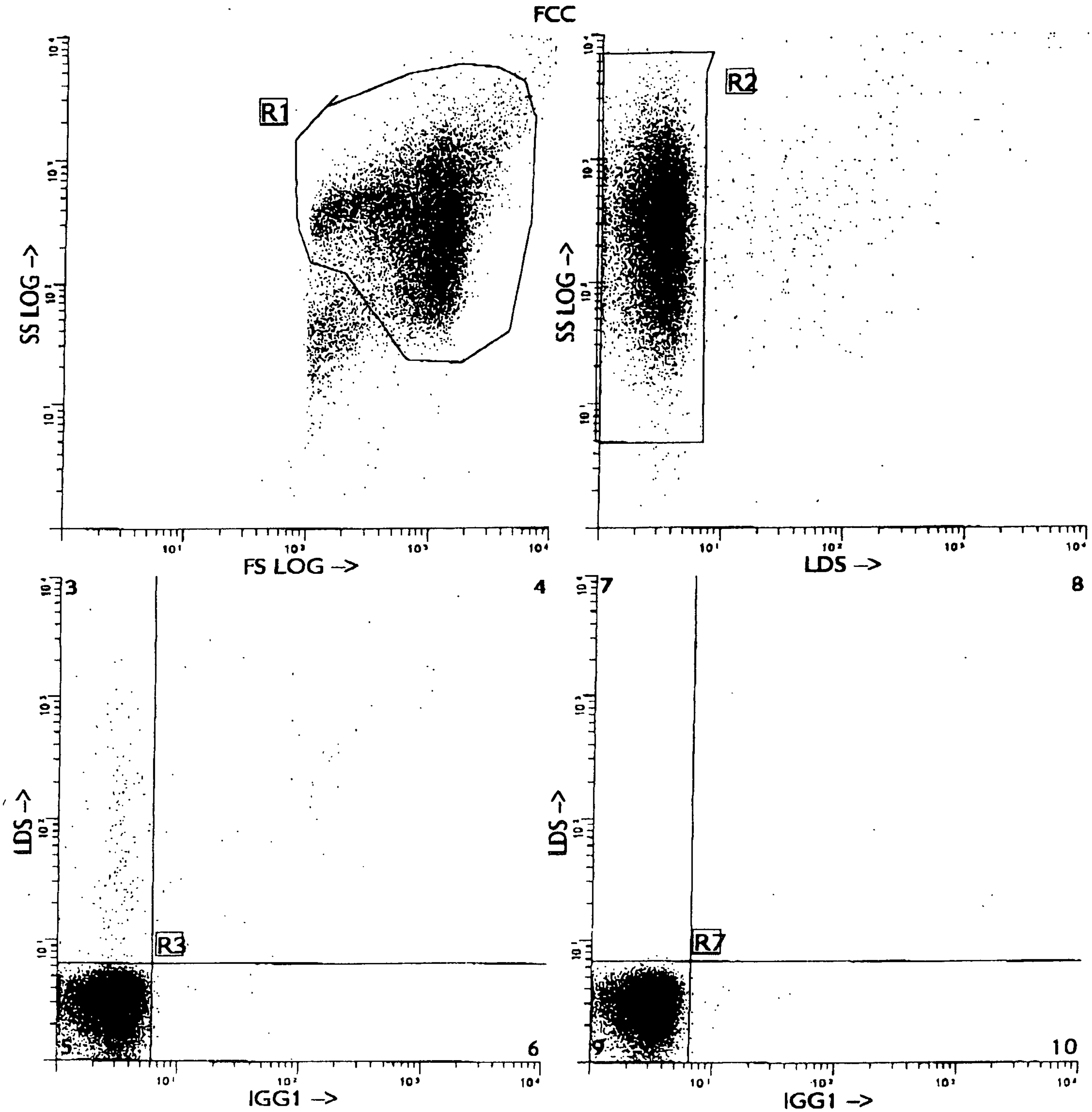

FCC
R1
SS LOG ->
FS LOG ->
R2
SS LOG ->
LDS ->
3
4
5
6
R3
LDS ->
IGG1 ->
7
8
9
10
R7
LDS ->
IGG1 ->

FIGURE 2

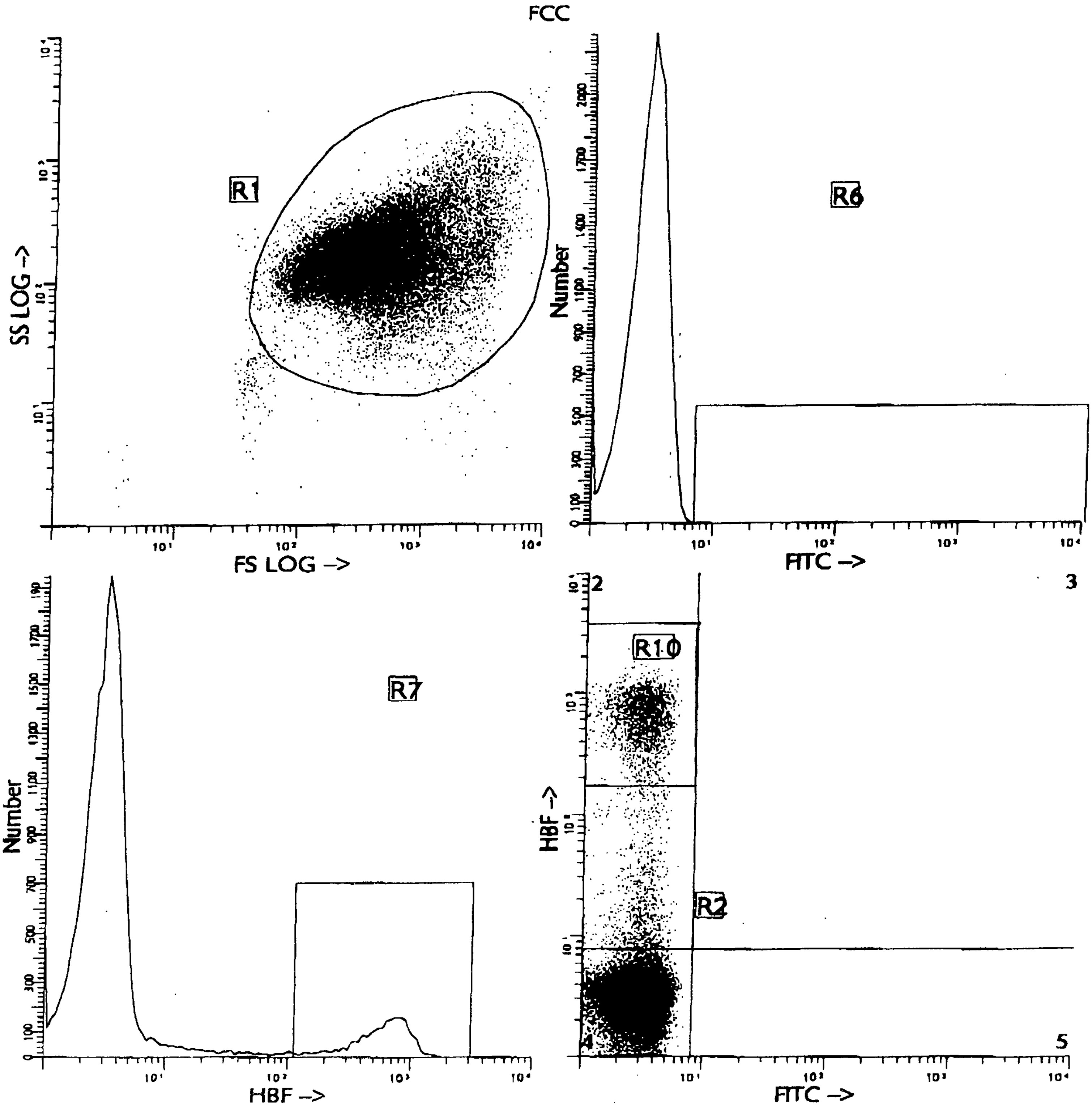
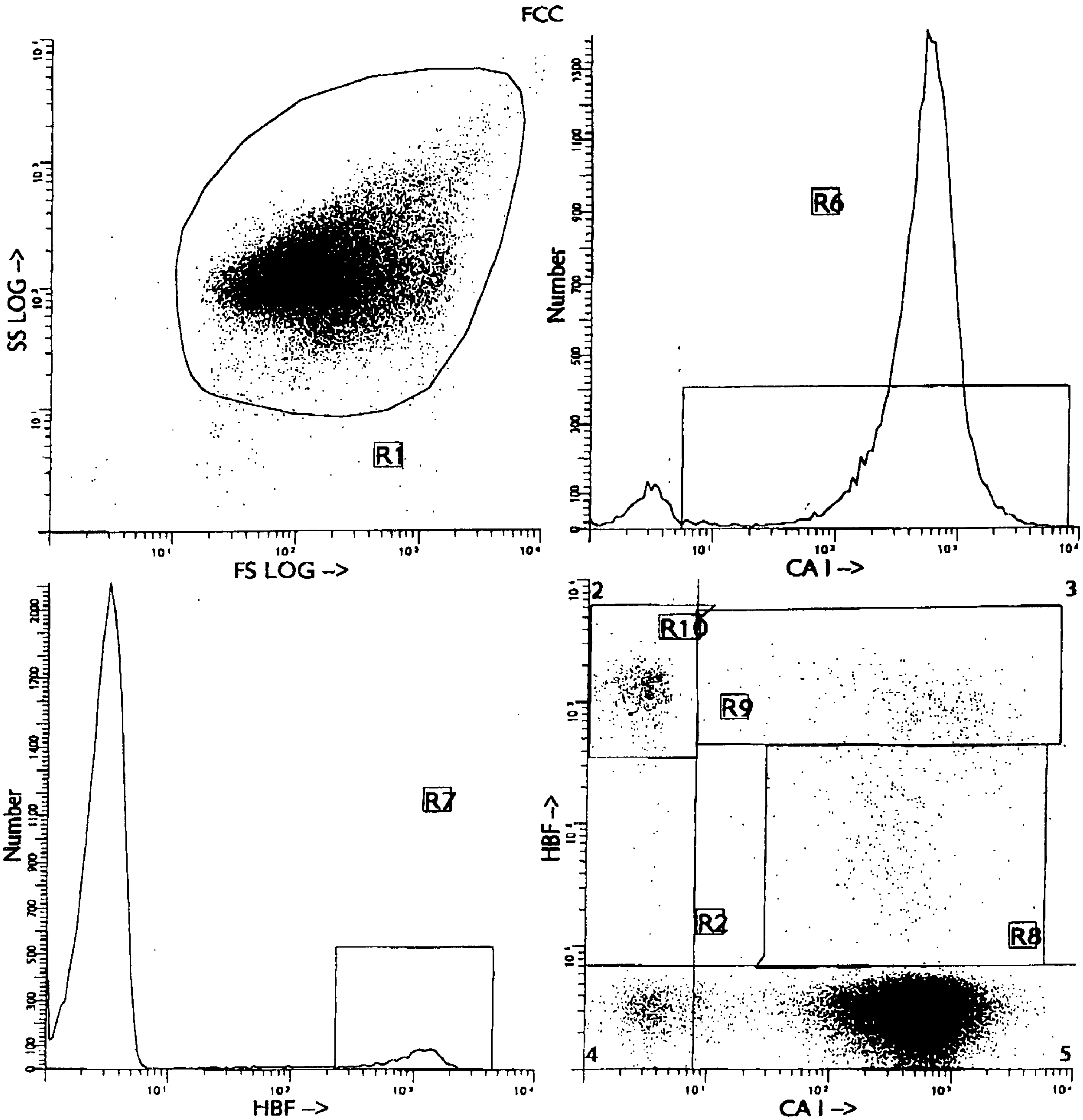

FCC
R1
SS LOG ->
FS LOG ->
R6
Number
FITC ->
R7
Number
HBF ->
R10
R2
HBF ->
FITC ->
2
3
4
5

FIGURE 3


FCC
SS LOG -->
FS LOG -->
R1
Number
CA I -->
R6
Number
HBF -->
R7
HBF -->
CA I -->
2
3
4
5
R10
R9
R2
R8